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Development and validation of a rapid HPLC method for the simultaneous determination of triethylenetetramine and its two main metabolites in human serum

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Abstract

A validated method for the determination of triethylenetetramine, a selective copper-chelator currently undergoing clinical trials for the treatment of diabetic heart failure, and its two major metabolites, N_1-acetyltriethylenetetramine and N_1,N_{10}-diacetyltriethylenetetramine in human serum using HPLC is reported. The method used 9-flouorenylmethylchloroformate chloride to label all three analytes. The fluorescence labeled analytes were then separated chromatographically using a reversed phase C18 column under a gradient elution program and detected spectrofluorometrically at 317 nm with excitation at 263 nm. Application of the method is demonstrated by pharmacokinetic measurement in one healthy volunteer taking the drug orally.
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1. Introduction

Triethylenetetramine (TETA) is a selective copper-chelator that has been used in the treatment of Wilson's disease for more than two decades and has demonstrated a low side-effect profile [1]. Recently, TETA has been shown to be effective in the treatment of cardiac complications in Type-2 diabetic subjects [2–4]. Two metabolites of TETA have been reported in human plasma and urine, being N_1-acetyltriethylenetetramine (MAT) and N_1,N_{10}-diacetyltriethylenetetramine (DAT) [5,6]. Detailed pharmacokinetic data for TETA, MAT and DAT are, however, yet to become readily available in the literature. In order to study pharmacology of TETA in humans, it is first necessary to develop a quantitative method to measure the drug and its metabolites in human serum.

There are several published procedures that describe the quantification of TETA in human plasma and urine [7–11]. However, none have been shown to be capable of the simultaneous determination of TETA, MAT and DAT in human serum, as the two metabolites, especially DAT, were only recently characterized, and these methods were mainly developed for the measurement of TETA alone. Furthermore, all the published methods have limitations. The method using 4-(1-pyrene) butyric acid *N*-hydroxysuccinimide ester (PSE) as a fluorescence-derivatizing agent is highly selective and sensitive but requires high temperatures for derivatization to occur [11]. Furthermore, we ourselves found that DAT could not be adequately labeled using PSE. The method using *o*-phthalaldehyde as a fluorescence-labeling agent with post-column derivatization reports the determination of TETA and MAT only, and the chromatograms obtained were complicated by the presence of many endogenous amino compounds [8]. Both methods and two further ones [9,10] that employed other fluorescence-labeling

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reagents, did not confirm that complete derivatization was achieved. Furthermore, the published conductometric method [7] has low sensitivity that was insufficient to meet the requirement for clinical drug monitoring in serum.

We herein present a validated and highly sensitive high pressure liquid chromatography (HPLC) method for detection and quantification of TETA, MAT and DAT in a single sample injection. We employed 9-flouorenylmethylchloroformate (FMOC) as the fluorescence labeling reagent; the derivatization procedure is simple and rapid and requires no heating. The method has been optimized to ensure complete derivatization by identifying fully labeled products using liquid chromatography–mass spectrometry (LC–MS) with an atmospheric pressure chemical ionization (APCI) interface. Derivatives are stable and well separated on a reversed-phase C18 column. The method has been applied to measure the concentration–time profile for parent drug and both metabolites in serum from a healthy volunteer who took a single oral dose of 600 mg of TETA dihydrochloride.

2. Experimental

2.1. Chemicals and reagents

Reference standards, TETA dihydrochloride (purity 99.93%), MAT trihydrochloride (purity 98.50%) and DAT dihydrochloride (purity 95.43%), were obtained from Carbo-Gen AG (Hunzenschwil, Switzerland), and the internal standard hexamethylenediamine dihydrochloride (HDA, 99% purity) and FMOC Chloride (97%) were from Sigma–Aldrich (St. Louis, MO, USA). Sodium tetraborate (99%) was from M & B Chemicals; ammonium acetate and ethylenediaminetetraacetic acid, disodium salt (EDTA) were from BDH chemicals; and HPLC grade acetonitrile was from Scharlau Chemicals. Water used in the preparation of buffers and standards was generated using an ELGA system (resistivity 18 MΩ cm^{-1}).

2.2. Preparation of standard solutions and reagents

Stock solutions of 100 mg/L of free base TETA, MAT and DAT were prepared separately by dissolution in water. A mixed standard (5 mg/L each) was prepared in pooled human drug-free serum. A 1 mmol/L HDA solution was prepared in water. Standard and stock solutions were stored at −80 °C. A 7.5 mmol/L FMOC solution was prepared in acetonitrile and stored at −80 °C and a 1 mmol/L EDTA solution was prepared and adjusted to pH 7.5 using 0.5 mol/L sodium hydroxide. Borax buffer was prepared by dissolving 10 g of sodium tetraborate in 200 mL of water (final pH was approximately 9.6). These solutions were stored at room temperature.

2.3. HPLC instrumentation

Analytes were measured using a Shimadzu (Shimadzu, Kyoto, Japan) HPLC system that consisted of a pump with four solvent lines, an online solvent degassing unit, an autosampler, a sample cooling unit with temperature controlled at 4 °C, a column oven and a fluorescence detector. Chromatographic separation was achieved by using a Luna 3 μ C18 (1) 150 mm × 3.0 mm analytical column (Phenomenex, Auckland, NZ) with a protective guard cartridge (Phenomenex) maintained at 25 °C. Linear gradient elution was performed using two mobile phases. Mobile phase A was 100% acetonitrile and B 10 mM ammonium acetate in Milli Q water (pH 7.0). The linear gradient program was: 0 min, 50% A; 7 min, 70% A; 7.5 min, 80% A; 17.0 min, 80% A; 19 min, 95% A; 23 min, 95% A; 25 min, 50% A; 35 min, 50% A. The overall run time was 35 min and overall flow rate was maintained at 0.5 mL/min. The excitation and emission wavelengths of the fluorescence detector were set at 263 and 317 nm, respectively. Ten microliters of sample was injected into the HPLC system. Data acquisition, peak integration with skim tangent, linear standard curve construction and analyte concentration calculations were achieved automatically using Shimadzu LC Solutions software (Shimadzu). Area ratios of analyte peak versus internal standard peak were used to construct standard curves. The data system performed the least squares linear regression analysis for each calibration curve with equal weighting for each point.

2.4. LC–MS instrumentation for method optimization

Optimization of the method parameters was done using a Shimadzu LC–MS equipped with a pump, online solvent degassing unit, auto sampler, sample cooling unit with temperature controlled at 4 °C and a 2010A single quadruple mass spectrometer with APCI interface. Blank serum spiked with standards was used in the optimization experiments. The chromatographic separation was achieved by using a Luna 5 μ C18(1) 250 mm × 4.6 mm analytical column (Phenomenex) maintained at 25 °C. The MS conditions were: CDL temperature, 200 °C; heat block, 300 °C; nebulizing gas, 2.5 L/min; interface voltage, 3.5 kV; CDL voltage, −20 V; polarity, positive; and Q-array voltage: DC 5.0 V, RF 150 V. Fluorescence-labelled analytes were identified using single-ion monitoring (SIM) detection set for the corresponding FMOC labelled species at the following molecular masses: 1035, TETA labelled with four FMOC groups; 855, MAT, labelled with three FMOC groups; 675, DAT labelled with two FMOC groups; and 562, HDA labelled with two FMOC; as well as 812, TETA labelled with three FMOC groups; 632, MAT labelled with two FMOC groups; 452, DAT labelled with one FMOC group; and 339, HDA labelled with one FMOC group. Data were acquired and processed using LC–MS Solutions software (Shimadzu).

2.5. Standard and sample preparation

A series of standards (0.0625, 0.125, 0.25, 0.75, 1.5 and 3 mg/L) was prepared from the 5 mg/L stock solution using human drug-free serum as diluent. A seven-point calibration curve was constructed in the range 0.0625–5 mg/L with each batch of samples. Three levels of quality control samples (run in duplicate) were included with each batch of samples prepared at 0.5, 1 and 4 mg/L for DAT; and 0.3, 1 and 4 mg/L for MAT and

TETA. Two blank samples (with and without HDA) of serum diluent were run with each batch of samples.

Samples and standards were treated as follows: to 25 μL of serum in an eppendorf tube were added 10 μL of 60 μmol/L HDA, 20 μL of 1 mmol/L EDTA (pH 7.5), 25 μL of water, 25 μL of sodium tetraborate solution, and 75 μL of acetonitrile. Samples were vortex mixed and allowed to stand for 10 min for deproteinization. They were then centrifuged at 10,000 × *g* for 3 min and supernatant (100 μL) added to HPLC glass vials. For derivatization, 100 μL of 7.5 mmol/L FMOC was added and vials were capped and shaken. Samples were stood for 10 min, then centrifuged at 4000 × *g* for 5 min. Supernatants were added to HPLC vials with glass inserts and centrifuged at 4000 × *g* for 5 min, then 10 μL of supernatants were injected into the HPLC apparatus.

2.6. Method validation

2.6.1. Precision, accuracy and recovery

The validation parameters linearity, accuracy, precision (intra- and inter-batch) and limits of detection were determined for each compound according to United States Food and Drug Administration (FDA) guidelines [12]. Linearity was assessed by a non-weighted least squares regression analysis. The acceptance criterion for each back-calculated standard was 15% deviation from the nominal value except LLOQ, which was set at 20%. Intra- and inter-batch precisions and accuracy were determined by analyzing 8 and 5 quality control samples, respectively, at each level. Recovery was measured at low, medium and high concentrations (0.3, 1 and 4 mg/L) by comparing blank serum spiked with analytes with deproteinized blank serum spiked with analytes.

2.6.2. Selectivity, sensitivity and stability

Selectivity is defined as the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. The analytes were measured in the presence of the following anticoagulants: citrate, EDTA, fluoride and heparin, each at concentrations of 1 mg/L. Analytes were also measured in the presence of varying degrees of hemolysis and lipemia (mild to gross) at concentrations of 1 mg/L. Medications commonly used by diabetic and heart failure patients were also processed by this method to determine whether there was any interference.

Tested medications were metoprolol succinate, metoprolol tartrate, glipizide, captopril, warfarin, diltiazem hydrochloride, felodipine, digoxin, furosemide, metformin hydrochloride, simvastatin, insulin, cimetidine, acetaminophen, salicylic acid, magnesium salicylate and paraaminobenzoic acid.

Short term stability was determined before derivatization by keeping quality control samples (in duplicate) at room temperature and at 4 °C, and analyzing these controls periodically throughout the day. The stability of the analytes was also assessed by a freeze-thaw procedure. Quality controls (in duplicate) were stored at −80 °C, thawed to room temperature, analyzed and stored again at −80 °C. The controls underwent three freeze-thaw cycles. Post-derivatization stability of analytes was determined by keeping quality control samples that have already been derivatized at room temperature and 4 °C and analyzing them periodically over a period of 52 h.

2.7. Measurement of TETA, MAT and DAT in serum samples of a healthy human volunteer

The suitability of the method for pharmacokinetic analysis was evaluated using serum samples from a healthy volunteer who was administered a single dose of 600 mg of TETA dihydrochloride orally. Serial serum samples were taken at time 0, 10, 20, 30, 45, 60, 75, 90 and 120 min and thereafter at 3, 4, 5, 6, 8, 10, 12, 16, 20 and 24 h after dosing. Samples were analyzed using the method described here.

3. Results and discussion

3.1. Optimum derivatization conditions

Optimum derivatization studies were carried out to ensure complete derivatization of all analytes and maximize peak area of TETA, MAT and DAT. Completely derivatized TETA, MAT and DAT are shown in Fig. 1.

The effects of temperature, pH and FMOC concentration on derivatization were studied. It was found that 25 °C was the optimal temperature where no incompletely derivatized analytes were detected. Temperatures of 60 and 80 °C caused the incomplete derivatization products to appear and increase. This was likely due to the formation of FMOC hydroxide (FMOC-OH), because higher temperature accelerates the hydrolysis of FMOC. It is therefore suggested that for complete derivatization to take place, FMOC is required rather than FMOC hydroxide. When incompletely derivatized, TETA has three FMOC groups attached and eluted as one peak in front of the fully labeled TETA peak; MAT has two FMOC groups attached giving two isomers which were observed as two closely related peaks on the MS chromatogram. These eluted earlier than the fully derivatized MAT as they have one less FMOC group. No partially derivatized DAT was observed. Fig. 2 shows a MS chromatogram of one injection with partially derivatized analytes.

Incomplete derivatization was also observed at concentrations of FMOC less than 7 mM. Using higher FMOC concentrations (>7 mM), complete derivatization of all analytes was achieved and no partially derivatized analytes were observed.

The derivatization reaction was facilitated by the addition of base. Sodium tetraborate buffer at pH 9.6 used as the reaction base gave the most intense peaks for all analytes. Higher pH (such as 11.4 and 12.9) would cause much smaller peaks for all analytes, mainly because at higher pH, FMOC was not stable and would be hydrolyzed to non-reactive FMOC-OH. Thus optimum conditions for complete derivatization were ambient temperature, a FMOC chloride concentration of 7.5 mM and a pH of 9.6. With such conditions, a clean chromatographic profile of completely derivatized analytes could be obtained (Fig. 3).

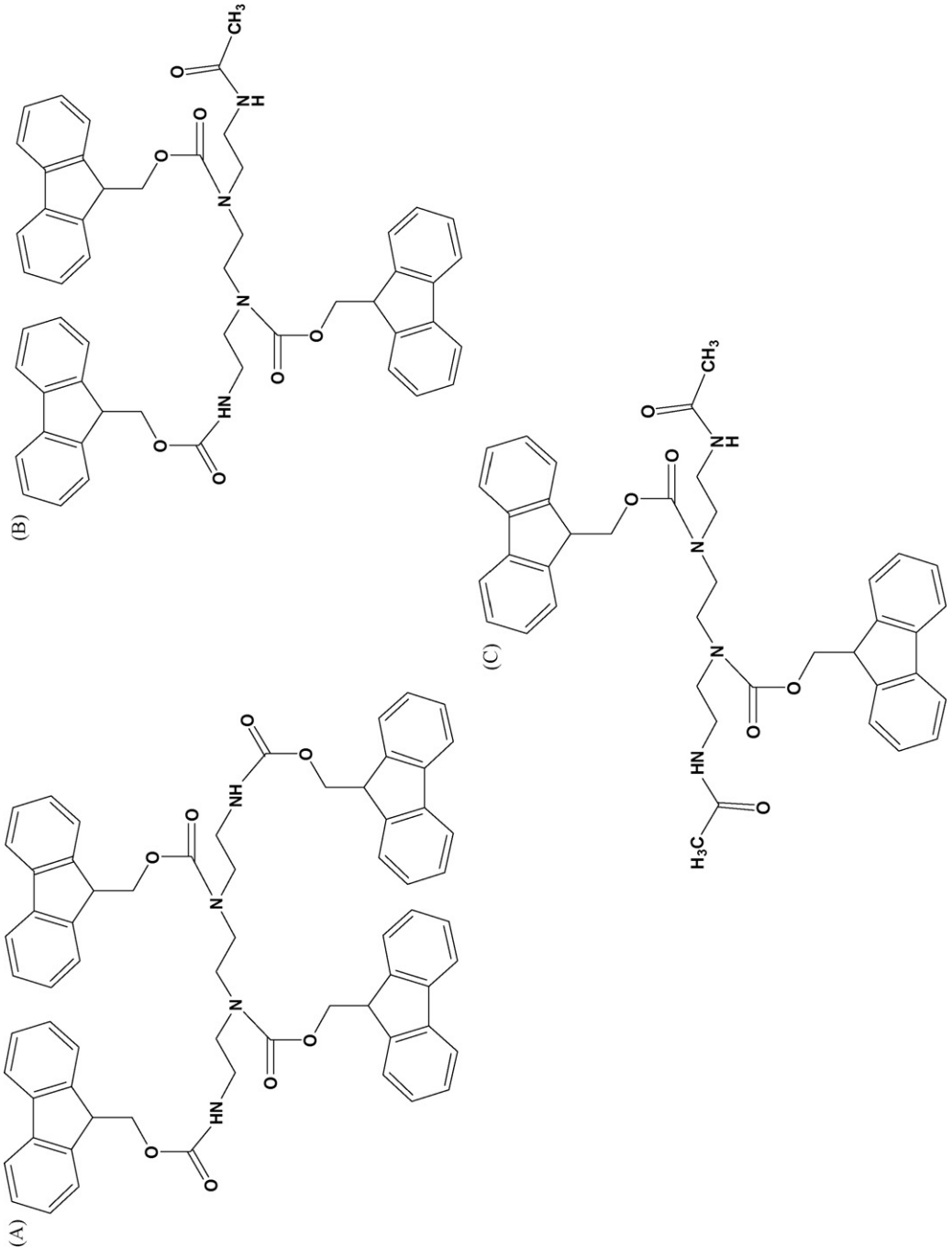

Fig. 1. Chemical structures of TETA (A), MAT (B) and DAT (C) completely labeled with FMOC groups.

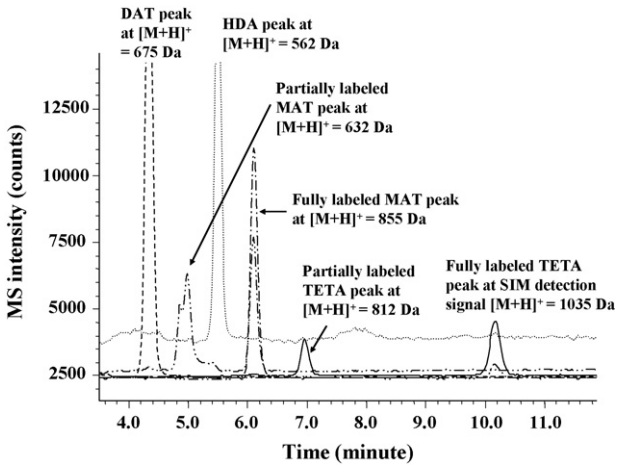

Fig. 2. LC–MS chromatogram showing co-existence of completely and incompletely FMOC labeled TETA, MAT and DAT.

3.2. HPLC chromatography

Using the optimized derivatization conditions and the HPLC settings described in the Section 2, all fully labeled analytes and internal standard peaks were well resolved (Fig. 4). After optimizing and finalizing the HPLC method, we purchased an ASI 620-P010 post-column flow splitter (ratio 3:1, ELM Scientific, Sydney, Australia). We diverted a quarter of the flow into the MS and confirmed the identities of those fully labeled analytes and the internal standard. Endogenous plasma components, such as putrescine, spermidine, spermine, acetylputrescine, acetylspermidine and acetylspermine, did not give any interference.

3.3. Validation

3.3.1. Selectivity

None of the medications tested interfered with this assay. EDTA, heparin, citrate and fluoride also did not interfere with the assay. Lipemic samples (mild to gross) could be used in this assay but hemolyzed samples (moderate to gross) were unsuitable.

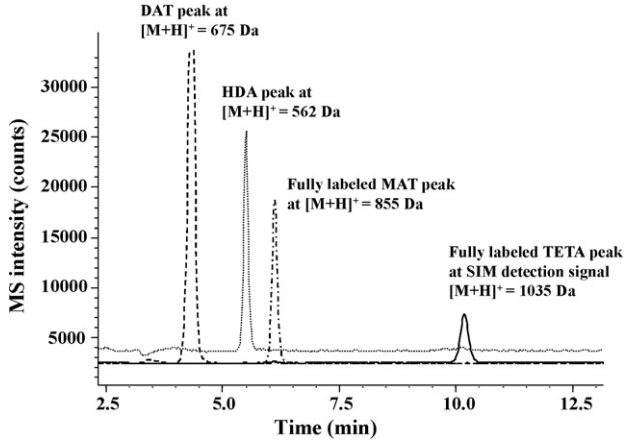

Fig. 3. LC-MS chromatogram showing separation and detection of completely FMOC labeled TETA, MAT and DAT.

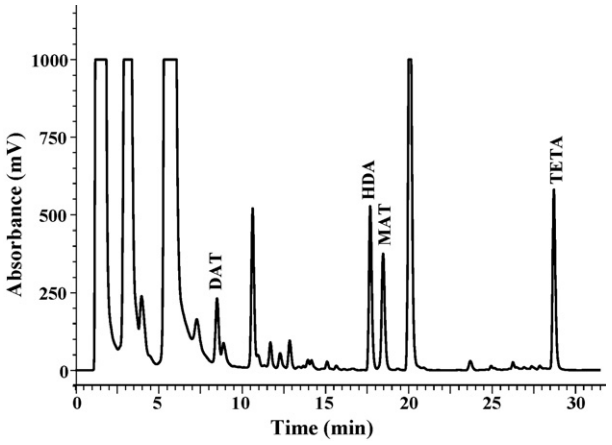

Fig. 4. HPLC chromatogram showing separation and detection of completely labeled TETA, MAT and DAT in a blank human serum sample spiked with 3 mg/L of each analyte and processed with the FMOC labeling procedure.

3.3.2. Stability

Results for stability showed that all derivatized analytes were stable in human plasma at room temperature for 24 h (concentrations after 24 h storage were within 10% deviation of the initial values). Derivatized analytes were also stable over the 52 h period when stored at 4 °C. After three freeze-thaw (−80 °C) cycles, the derivatized analytes were also stable and concentra-

Table 1
Precision results of method validation

Spiked Concentration (mg/L)	Concentration found (mean ± S.D.) (mg/L)	Precision	Relative Error (bias, ±%)
Intra batch ($n = 8$)			
DAT			
0.5	0.57 ± 0.02	3.5	14.0
1.0	1.08 ± 0.03	2.8	8.0
4.0	4.59 ± 0.32	7.0	14.8
MAT			
0.3	0.32 ± 0.01	3.1	6.7
1.0	0.96 ± 0.03	3.1	4.0
4.0	3.95 ± 0.27	6.8	1.3
TETA			
0.3	0.32 ± 0.02	6.3	6.7
1.0	1.02 ± 0.06	5.9	2.0
4.0	4.35 ± 0.31	7.1	8.8
Inter batch ($n = 5$)			
DAT			
0.5	0.51 ± 0.05	9.8	2.0
1.0	1.05 ± 0.11	10.5	5.0
4.0	4.31 ± 0.30	7.0	7.8
MAT			
0.3	0.28 ± 0.03	10.7	6.7
1.0	0.90 ± 0.04	4.4	10.0
4.0	3.89 ± 0.17	4.4	2.8
TETA			
0.3	0.30 ± 0.03	10.0	0.0
1.0	0.89 ± 0.04	4.5	11.0
4.0	3.92 ± 0.43	11.0	2.0

tions (low, medium and high) were also within 10% (deviation) of the initial values.

3.3.3. Calibration curve

Calibration curves were linear over the concentration range of 0.125–5 mg/L for MAT and DAT and 0.0625–5 mg/L for TETA with a correlation coefficient of 0.99 or better and an intercept value not statistically different from zero. The lower limit of qualification (LLOQ) for DAT and MAT was 0.125 mg/L and that for TETA was 0.0625 mg/L. The upper limit of qualification (ULOQ) for all analytes was 5 mg/L.

3.3.4. Precision, accuracy and recovery

Intra batch precision ($n = 8$) and inter batch precision ($n = 5$) were less than 7.1% and 11.0% (deviation), respectively, for all analytes. Intra batch and inter batch relative errors (deviation) were less than 14.8% and 7.8%, respectively, for all analytes at the described concentrations. Results are listed in Table 1. The recovery range of all concentrations (low, medium and high) was 91–108%.

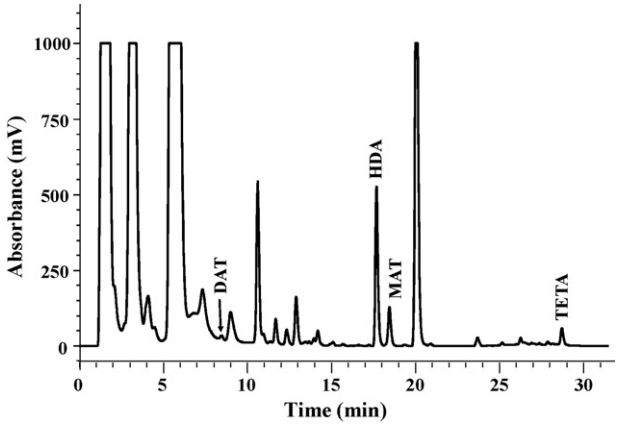

Fig. 5. A representative HPLC chromatogram showing detection of TETA, MAT and DAT in 5 h post-dose human serum.

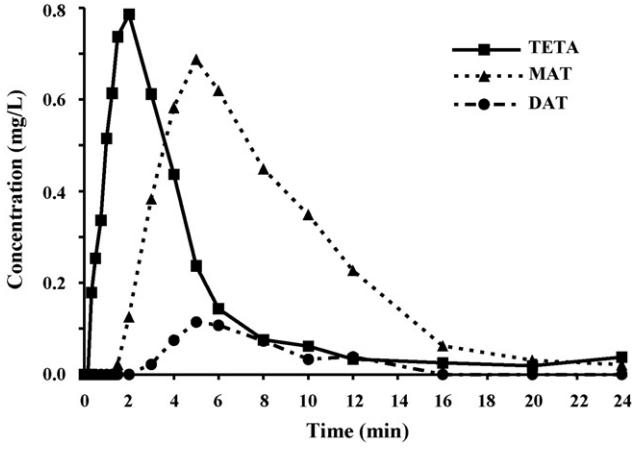

Fig. 6. Twenty-four hour time-concentration profiles of TETA, MAT and DAT in serum of a healthy volunteer who took 600 mg TETA dihydrochloride orally.

3.4. Application

The described assay has been successfully employed to quantify all three analytes in human plasma samples following the oral administration of a single 600 mg dose. A representative chromatogram of analytes detection is shown in Fig. 5. The resulting time–concentration profile is shown in Fig. 6. The data were analyzed using WinNonLin v5.2 (Pharsight Corp., Mountain View, CA, USA). A non-compartmental model was used to calculate pharmacokinetic parameters. C_{max} values were 0.79, 0.62, 0.11 mg/L; T_{max} values were 2, 5 and 5 h; $t_{1/2}$ values were 2.5, 5.3 and 10.8 h; and $AUC_{0\text{-}24}$ measurements were 3.0, 5.2 and 1.0 mg-h/L for TETA, MAT and DAT, respectively.

4. Conclusion

A validated HPLC method with fluorescence detection has been described for the simultaneous quantification of TETA, MAT and DAT in human serum. Sample preparation involves fluorescence derivatization using FMOC at room temperature. The accuracy and precision are good and it is sensitive with relatively low LLOQ values. Therefore, this method is suitable for the pharmacological studies of TETA and its metabolites in humans, of which an example has been provided.

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